Caenorhabditis elegans: The Search for *srf-6* Mutations

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Abstract

Parasitic nematodes may evade host immune systems by changing surface composition during infection. *C. elegans* is a free-living nematode with a conserved development and cuticle structure utilized as a parasitic model. *srf-6* mutant worms fail to make surface antigen switches that occur facultatively in the wild type. The whole genome sequences of three *srf-6* mutants were determined to identify the DNA sequence corresponding to *srf-6*. This project's objective was to find and confirm *srf-6* mutations in the genome sequence by re-sequencing the candidate gene in mutant DNA.

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Chapter 1. Introduction

Model System

Since Sydney Brenner's introduction of *C. elegans* in 1974 (Brenner, 1974), the organism has been used as a model to study development and the nervous system. It also has potential to help explain disease pathogenesis, suggest new therapeutic treatments, and allow whole-animal target and drug screening (Kaletta and Hengartner 2006).

C .elegans is a small free-living nematode whose phylum consists of both parasitic and free living worms. *C. elegans'* body is uniquely suited to research as it is transparent, further facilitating study of its functions. Stocks of *C. elegans* are easy to maintain in petri dish cultures on agar substrates, requiring only *E. coli* bacteria lawns to sustain a population.

The lifespan of a worm is around two weeks, but its generation time is around three and a half days, allowing for rapid genetic analysis. Most *C. elegans* worms are hermaphrodites and are capable of producing about 300 offspring before they run out of sperm. Males are produced by spontaneous non-disjunction at a frequency of 0.1%. If a male individual is present on the plate to mate with hermaphrodites and fertilize their eggs, cross progeny can be produced. Because male sperm are used preferentially over hermaphrodite sperm, the result is a 50-50 mix of males and hermaphrodites in the cross progeny. Thus as a genetic system, *C. elegans* offers convenient control over reproduction; homozygous strains can be

maintained as hermaphrodites, and genetic markers can be transferred between strains by crossing hermaphrodites with males.

C. elegans was the very first multi-cellular animal to have its genome sequence determined (*C.elegans* Consortium, 1998). The *C. elegans* genomic DNA sequence is composed of 100 million base pairs distributed on six different chromosomes, which are labeled from I-V and X.

There are five stages in the development of *C. elegans*, shown in **Figure 1**. The stages include the embryo, L1 through L4, and adult, with an additional facultative dauer larva stage between L2 and L4 that is dependent on the environmental conditions (Hu, 2007). The dauer stage is an alternative to the L3 stage that develops as a larval response to three different parameters: population density, food supply and temperature. When food is scarce and dense populations of *C. elegans* are present, L2 larvae molt into dauer larvae, which have no body openings, a very slender body, a tough cuticle, accumulations of fat in the intestinal cells, and a remodeled pharynx (Hu, 2007). These alterations allow the dauer larvae to endure for extended periods of time until conditions allow for normal development.

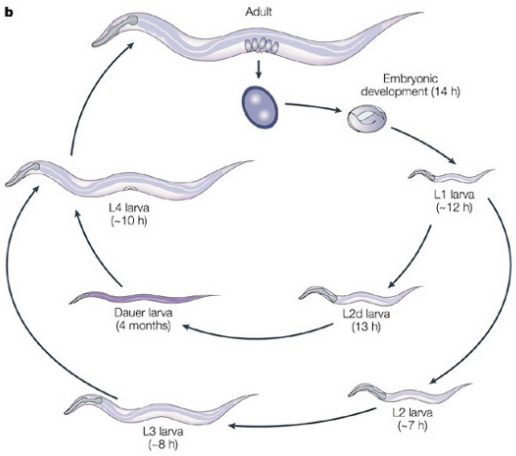

Figure 1: The Five Stages in the Development of *C. elegans* at 25°C
(Adapted from Jorgensen and Mango Nature Reviews Genetics 3:356-369).

Chemosensation

C. elegans uses chemosensation to detect and respond to olfactory and water soluble signals in its environment. Chemosensation is probably of great importance to the organism, as 5% of the coding genome in a *C. elegans* worm is involved in chemosensation (Bargmann, 2006). Furthermore, 32 out of the 302 neurons are devoted to chemosensation (Hobert 2010). With properly functioning chemosensation, *C. elegans* can find food, avoid dangerous environmental conditions and pathogens, and search for a mate. Chemosensation can also signal for behavioral changes and developmental changes such as the dauer stage in the *C. elegans* life cycle. Chemosensation can even be used as a signal for *C. elegans* to change its surface composition, a trait it shares with related parasitic nematodes. In parasitic nematodes, changes in surface composition can be for reasons such as avoiding pathogenic infections, or evading a host's immune system (Olsen et al, 2007).

Cuticle structure and surface composition

The surface composition of the *C. elegans* cuticle and that of its parasitic relatives does not remain static during its lifecycle. Post embryonic development of *C. elegans* involves molting its cuticle during the transition between each stage for a total of four times. Molting consists of de novo synthesis of a new cuticle and shedding of the old one. Molting is accompanied by a sleep-like period of lethargus, during which worms do not feed or move about. This is a fundamental mechanism in nematode development. In *C. elegans* the cuticle structure is distinct in three stages: the L1, dauer, and adult stage cuticle. The L2-L4 cuticles are all similar in structure, suggesting that these molts are isomorphic. However, in the other molts, changes in cuticle structure occur, suggesting that these molts are metamorphic. The cuticle, shown in **Figure 2**, is composed of multiple layers: an outermost layer composed of glycoproteins, an insoluble matrix, osmiophilic epicuticle, and several inner structural layers composed of collagen proteins (Page and Johnstone, 2007).

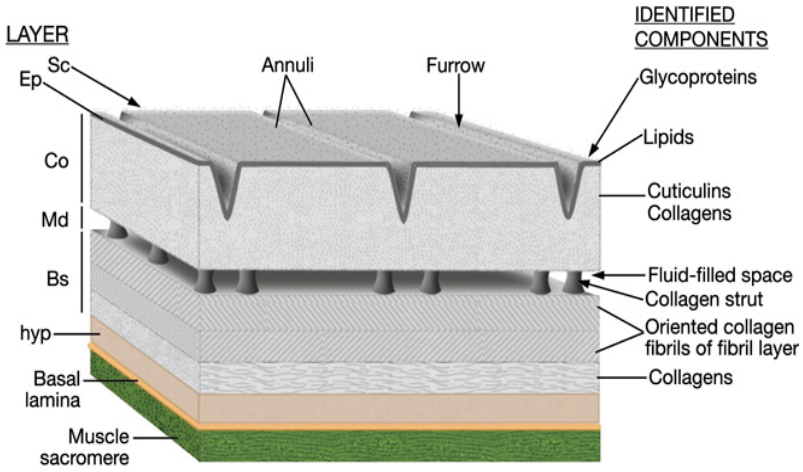

Figure2. Layers of the Adult *C. elegans* Cuticle. (Sc) Surface coat; (Ep) Epicuticle; (Co) Cortical zone; (Md) Medial zone; and (Bs) Basal zone (Adapted from Blaxter and Robertson, 1998).

Changes in surface composition during nematode development

Politz and co-workers have used surface-specific antibody probes to identify *C. elegans* mutants that have altered surface composition. The *srf* (SuRFace) class of mutant phenotypes includes deficits in surface components that expose normally hidden components (Politz et al 1990; Link et al., 1992). Several of these genes encode genes involved in biosynthesis of glycoproteins, suggesting that the mutant phenotypes result from changes in antigenic surface (Gravato-Nobre et al., 2011) . Other *srf* genes, such as *srf-6*, result in changes in the timing of display of stage specific epitopes when they are mutated (Grenache et al., 1996; Olsen et al., 2007).

The *srf* class of mutant genes, found in *C. elegans,* causes a phenotype of changed surface reactivity to antibody or lectin reagents. Several of these *srf* mutants have been isolated and categorized into three groups. The first group, (*srf-1, srf-2, srf-3,* and *srf-5*) expresses a phenotype at the cuticle surface that arises from

the altered exposure of antibody epitopes or lectin-binding sites (Politz et al. 1987, 1990; Link et al. 1988, 1992). The second group, (*srf-4, srf-8,* and *srf-9*) is composed of the pleiotropic *srf* loci that contain defects in both internal and external structures (Link et al. 1992). These defects are exhibited in the form of protruding vulvae and a low-penetrance multivulval phenotype, male infertility, distorted copulatory bursae and abnormal spicules, defects in gonadal morphology, distortion of body shape, and uncoordinated movement. The third group (*srf-6*) affects the timing of surface antigen expression. The *srf-6* mutation causes an antigen that is normally present only on L1 larvae to be expressed at later larval stages (Hemmer et al., 1991; Grenache et al, 1996).

Because of the similarities of the cuticle development and structure within the nematode phylum, it is possible that there are analogs of the *srf* class of genes in parasitic nematodes. In the case of parasitic nematodes, these genes could allow for evasion of the immune system, which relies on the identification system of antibodies and effector cells to combat parasitic infection. Furthermore, studies have shown that several parasitic worm species such as *Trichinella spiralis* are also able to change their surface composition within stages of development, augmenting their ability to evade the immune system (Philipp et al., 1981; Jungery et al., 1983; Bell R.G et al., 1979).

The surface composition of parasitic nematodes such as *Ascaris* is similar to that of *C. elegans*. The cuticle contains collagens, cuticlin, and surface associated proteins along with glycoproteins. In fact, some important features of the nematode cuticle, such as cuticlin, were first isolated in parasitic nematodes like *Ascaris*

(Kanaya and Fujimoto, 1973). Of particular note is that parasitic nematodes also have surface antigens, which are specific to each stage of development, a trait they share with *C.elegans* (Philipp et al, 1981). The parasitic antigens can be released into the environment, which has been speculated to be a defense mechanism against an antibody mediated immune response from the host (Politz and Philipp, 1992). However, the molecular mechanisms behind the stage specific antigen expression in the parasitic nematodes remain unknown.

Project Purpose

The long-range goal of this project is to understand how *srf-6* functions to control surface antigen composition in *C. elegans.* The study of *srf-6's* mutant phenotype indicates that this gene plays multiple roles in the organism; for example, mutations in *srf-6* affect dauer formation, chemotaxis, and the surface composition of *C. elegans* (Olsen, 2007). However, the study of the *srf-6* gene has been confounded by the fact that the genomic sequence of *srf-6* has not been determined. Knowledge of the molecular identity of the gene product encoded by *srf-6* can potentially offer a hypothesis to explain its involvement in several distinct processes, and allow experimental tests of such a hypothesis to be performed. The goal of this project is to identify the genomic sequence encoding *srf-6* in *C. elegans.*

Potential Disease Model for Parasitic Worms

C. elegans and nematodes as a phylum have similar characteristics that could facilitate the use of *C. elegans* as a disease model. *C. elegans* has many advantages as a model for parasitic nematodes. One is that it is much easier to culture than

parasitic nematodes, which require co-culturing with one or more host organisms in order to study a complete life cycle, whereas the entire life cycle of C. elegans can be seen on a single petri dish. All nematodes undergo four different postembryonic developmental stages. The general structure of the cuticles of different nematodes is similar, further allowing *C. elegans* to potentially be a model for other nematode organisms (Grenache et al., 1996).

Using *C. elegans,* interactions can be studied within the context of the whole organism instead of basing results on isolated interactions. This is advantageous because it allows the actual behavioral response of the animal to be monitored. For example, a drug that is used to regulate several targets can easily be studied in *C. elegans* to determine the *in vivo* action. *C. elegans* has bridged the gap between *in vitro* assays, which are limiting in terms of their correspondence to *in vivo* activity, and mammalian models, which can be complex and expensive (Kaletta and Hengartner, 2006).

As mentioned previously, *C. elegans* worms molt between each of the postembryonic stages (L1-L4); at each molt a new multi-layered cuticle is formed. During these molts, *C. elegans* experiences cuticle structure changes such that there is a noticeable difference between L1, L2-L4, and the adult worms. The difference between L2-L4 worms is more difficult to detect however, because they have similar fibrous layers (Kramer, 1997). This is an important characteristic because similar stage-specific surface compositions are also seen in parasitic nematodes. Surface-associated antigens are located on the surface of *C. elegans* however it is unknown whether they are distributed among other layers of the cuticle as well.

Surface antigen switching restricts certain surface molecules to a specified time or stage in *C. elegans* postembryonic development. Although it is not a parasite, evidence for surface antigen switching has been found in *C. elegans*. In immunofluorescence testing using a monoclonal antibody, M37, *srf-6* mutants showed an altered display of a stage-specific surface epitope. Also by using immunofluorescence, it was shown that the wild-type *C. elegans* displayed the same phenotype as *srf-6* mutants when grown in the presence of an extract of spent liquid nematode culture medium. This phenomenon has been called Inducible Larval Display (ILD). ILD occurs when wild type worms are induced to display an L1 surface epitope at later larval stages when grown on extract of spent culture medium (Grenache et al., 1996). Similarly, in parasitic nematodes, surface composition is not only altered between stages but can also change in response to environmental signals; for example, during a transition to a new host or host tissue (Politz and Philipp, 1992). Thus manipulating surface composition at different stages could allow nematodes to escape immune attacks that target stages present early in infection. Understanding the mechanism by which surface antigen switching occurs would be very beneficial in explaining nematode responses to external conditions. It could also help in devising a strategy to prevent parasites from evading immune attack.

C. elegans genetics allows easy isolation and analysis of mutants. Through the use of transposon tagging and restriction fragment length polymorphism (RFLP) mapping of *C. elegans,* genes whose products are unknown is possible. Among the *srf* genes, *srf-3* set the precedent for this practice when it was cloned, as its product was

unknown prior to cloning (Höflich et al., 2004).

In a study of surface antigens in *C. elegans*, ethyl methanesulfonate (EMS) was used to mutagenize worms, whose progeny were screened using anti-cuticle antibodies to find mutants with surface alterations (Politz et al 1990). The mutations were then studied using genetic mapping and complementation data. EMS is a mutagen used to produce random mutations in genetic material by nucleotide substitution that primarily results in point mutations. Therefore the mutations that were found, in the *srf* loci (*srf-2* and *srf-3*), were predicted to be single-base substitutions. This gives evidence to the idea that small genetic changes may be the cause of distinct changes in surface antigenicity, as a single gene or cluster of genes could control the expression of these surface antigens. If this is true, the mutations do not need to accumulate over long periods to become significant. Instead, mutants such as *srf-2*, *srf-3*, and now *srf-6* could serve as feasible models in understanding the mechanisms of antigen expression regulation (Politz and Philipp, 1992).

In *C. elegans*, surface molecules appear to be shed from the surface of worms stained with monoclonal antibody M37 (Politz and Philipp 1992; Politz unpublished). This supports the earlier idea of antigen shedding by parasitic worms as a defense against host immune attack (Silberstein and Despommier, 1984). The discovery and understanding of mutants that do not experience antigen shedding could allow the direct identification of the gene and gene products responsible for this defense mechanism (Politz and Philipp, 1992). Although these studies were done with *T. spiralis*, similar experimentation can be performed using *C. elegans* due

to high degrees of similarity in environmental behavior and surface composition changes between parasitic worms and the free-living *C. elegans* nematode.

Research Course of Action

In order to identify the DNA sequence corresponding to *srf*-6, we will use the existing information from the previously conducted whole genome-sequencing project (Politz unpublished). Variant sequences from the three *srf-6* mutant strains will be analyzed using Galaxy to determine whether mutations in a specific gene are found in all three mutant DNA sequences. This search will be guided by the fact these *srf-6* mutations fail to complement and all map to chromosome II. Once a candidate gene is identified, the mutant genes will be re-sequenced for confirmation. In order to do this, polymerase chain reaction (PCR) amplification will be performed using oligonucleotide primers that flank each mutation. The PCR product will be inserted into a plasmid vector in one step using the TOPO® TA Cloning® Kit for Sequencing. Samples containing the inserts will then be sent out for sequencing.

Chapter 2. Materials and Methods

Genomic DNA Prep

C. elegans genomic DNA was prepared according to Sarin et al. (2002) with modifications by Mark Alkema (University of Massachusetts Medical School-Worcester, personal communication).

Galaxy

Galaxy (https://usegalaxy.org/) is an open source, web-based platform for data intensive biomedical research. The use of Galaxy to identify *C. elegans* mutations in whole genome sequencing data has been described (Minevich et al 2012). Whole genome sequencing (WGS) should allow researchers to pinpoint genetic differences between wild type and mutant strains of *C. elegans* more easily than by using other techniques. Galaxy helps simplify the bioinformatic analysis of the relatively short reads generated by second generation sequencing platforms by providing users with information regarding what data to operate on, what steps to take, and what order to do them in.

In work done by Samuel M. Politz and Jennifer Pulkowski (unpublished), the workflow described in Figure 3 of Minevich et al (2012) was followed to separately align whole genome sequences of *srf-6(yj13)*, *srf-6(yj15)*, and *srf-6(yj41)* with the *C. elegans* reference genome sequence. The GATK Unified Genotyper plug-in identified variant sequences in each mutant that differed from the wild type sequence. To remove variants that were shared in common by all of the mutant strains, the GATK Select Variants plug-in was used to subtract the union of two mutant variant sets

from the third one. This was repeated for all three possible pairwise subtractions to produce a set of mutant-specific variants.

These variant lists were analyzed by the Galaxy snpEff Variant effect predictor, which identifies the type of mutation according to which type of sequence it affects (intron, intergenic region, or exon), using the protein coding regions predicted from the annotated *C. elegans* genome sequence.

In our project, we used Galaxy's in-silico complementation program to sort through the mutations found previously by Politz and Pulkowski (unpublished). In-silico complementation testing compared the effects of the mutations from all three strains by producing a list of mutations that were found in the same gene in at least two of the three analyzed *srf-6* mutants. This list not only showed which alleles contained mutations, but also indicated the type of genetic sequence affected, the type of mutations found, their exact position based on the genome sequence, and the amino acid change predicted to result from each mutation.

Primer Blast

Primers were designed using NCBI's Primer Blast web site (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify base pair fragments from *Caenorhabditis elegans* reference sequence Bristol N2 Chromosome II (NC_003280.10). Eight primers were created, including a forward and reverse primer pair for *yj41*, *yj15*, and two for *yj13* (*yj13*A and *yj13*B). The primer search range covered approximately a 2,000 base pair region flanking the location of each mutation identified previously using Galaxy. The primer criteria for temperature for the minimum, optimum, and maximum temperatures were 57^0C, 60^0C, and 63^0C,

respectively. The product length of these primers was set between 200 and 700 base pairs in length, to give a product that could be completely sequenced from either end. To make up a 20 µM stock from the dry primers, 1 mL of water (dH_2O) was added to the primer vials.

Polymerase Chain Reaction

The Techne Progene PCR Thermal Cycler with Heated Lid was used to complete polymerase chain reaction (PCR). A master mix of components was created on ice using a microcentrifuge tube containing 25 µL 10X High Fidelity PCR Buffer, 5 µL 10 mM dNTP mixture, 10 µL 50 mM MgSO4, 10 µL Primer mix (20 µM each), 1 µL Platinum® Taq High Fidelity, and 199 µL sterile, distilled water, in this order. For each mutant strain, separate PCR tubes were then used containing 49 µL of the master mix, previously created, and 1 µL Template Genomic DNA for each mutant strain (*srf-6(yj13), srf-6(yj15), or srf-6(yj41)*). The PCR tubes were then run in the thermal cycler using the following program: 94°C for 1 minute with 30 cycles of the following: 94°C for 22 seconds (denature), 55°C for 22 seconds (anneal), 68°C for 1 minute (extend), followed by a holding step of 4°C for 99 hours and 59 minutes. The samples were kept at 4°C until they were needed for gel electrophoresis.

Gel Electrophoresis of PCR Samples

Following PCR amplification, gel electrophoresis of PCR products was performed using a 2% agarose gel. To 1 g of type LE agarose, 49 mL of 0.5 X TBE buffer was added. The mixture was then microwaved for 1 minute until all of the

agarose dissolved, making a clear solution. After cooling to approximately 50° C, the solution was poured into a gel caster with the comb, and allowed to cool for 20 minutes. After the gel had sufficiently cooled, the comb was removed and the gel was placed into the electrophoresis unit (Hoefer HE33 Mini Horizontal Agarose Electrophoresis Unit). Running buffer was then poured into the unit until it just covered the agarose gel. To be able to track the samples on the gel, 10 µL of loading dye (0.2% Bromophenol blue (BPB) and 25% glycerol) was added to 10 µL of each sample. The samples were mixed with 10 µL of λ DNA/*HindIII* Marker at .5µg/mL at 10x and 10 µL of the mixture was then loaded into the wells. The gel was run at 50-60 V until the bromophenol blue tracking dye migrated down two thirds the length of the gel. The gel was immediately post-stained with Ethidium Bromide (12.5 µL EtBr at 10 mg/mL dissolved in 250 mL 0.5 X TBE buffer) and placed on a bench top shaker for 45 minutes. An image of the gel was taken using Biorad Gel Documentation Apparatus and the camera software Quantity One.

Cloning

To clone the PCR products, the TOPO® TA Cloning ® Kit for Sequencing was used. To set up the TOPO® Cloning reaction, reagents were added to an eppendorf tube in the following order: 4 µL Fresh PCR Product, 1 µL Salt Solution, and 1 µL TOPO® vector to make up a final volume of 6 µL. The reaction was mixed gently and incubated for 15 minutes at room temperature (22°C to 23°C) and then placed on ice. Into a vial of One Shot® Chemically Competent *E. coli*, 2 µL of the TOPO® Cloning reaction was added and mixed gently. The cells were incubated on ice for 15 minutes, heat-shocked for 30 seconds at 42°C without shaking and immediately

transferred to ice. To the vials, 250 µL of room temperature S.O.C. medium was added. The tubes were capped tightly and shaken horizontally (200 rpm) at 37°C for 1 hour. On a pre-warmed 100 mm selective plate (LB-Amp with a final Amp concentration of 50µg/mL), 50 µL of each transformation was spread and incubated overnight at 37°C. To test for the effectiveness of Amp selection, an Amp-sensitive strain of *E. coli*, OP-50, was streaked on a separate LB-Amp plate. Nothing grew on this plate overnight.

Transformed Cell Liquid Cultures

The following day the plates were inspected. To 12 separate sterile culture tubes, 2 mL of LB stock containing 50 µg/mL ampicillin (4 µL Amp, stock concentration of 25 µg/ mL) was added. Three colonies were picked from each plate containing the transformed *E. coli* and placed in separate culture tubes. Then they were incubated overnight on a tube rotator at 37° C.

Plasmid Miniprep

Plasmids were isolated from transformed cells using the Qiagen Spin MiniPrep. The overnight culture samples were transferred to 1.5 mL microfuge tubes and spun down in the microfuge for 10 minutes. The supernatant was removed using narrow tips on the pipettor, and the pellet was re-suspended in 250 µL Buffer P1. The cells were vortexed and once fully re-suspended, 250 µL of Buffer P2 was added, which resulted in the samples turning blue, and the tubes were inverted gently 4-6 times. Afterwards 350 µL of Buffer N3 was added and the blue samples reverted to clear. The tubes were immediately but gently inverted again 4-6

times, and then they were centrifuged for 10 minutes at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. The supernatant was added to 12 QIAprep spin columns by pipetting, which were then connected to the vacuum manifold. The switch on the vacuum source attached to the manifold was turned on to draw the solution through the QIAprep spin columns, and then switched off. To wash the QIAprep spin columns, 0.5 mL of Buffer PB and then 0.75 mL Buffer PE were added, switching on the vacuum source before each addition and after the solution moved through the column, switching off the vacuum source. The QIAprep spin columns were then transferred to microcentrifuge tubes and centrifuged for 1 minute. To elute the DNA, the QIAprep columns were placed in clean 1.5 mL microcentrifuge tubes and 50 µL Buffer EB (or water in some cases) was added (10mM Tris-Cl, pH 8.5) to the center of the QIAprep spin columns. The tubes were incubated at room temperature for 1 minute, centrifuged for 1 minute, and the supernatant was kept.

Gel Electrophoresis of Plasmid Minipreps

Following the plasmid miniprep, agarose gel electrophoresis was performed. To 0.5 g of agarose, 49 mL of 0.5 X TBE buffer was added to make a 1% agarose gel. The mixture was then microwaved for 1 minute until all of the agarose dissolved, making a clear solution. The solution was allowed to cool to approximately 50° C before pouring. The solution was poured into a gel caster with the comb, and allowed to cool for 20 minutes. After the gel had sufficiently cooled, the comb was removed and the gel was placed into the electrophoresis unit. Running buffer was then poured into the unit until it just covered the agarose gel. To be able to track the samples on the gel, 2 µL of loading dye (0.2% Bromophenol blue (BPB) and 25%

glycerol) was added to 5 µL of each sample. The samples were then loaded into the wells along with 5 µL λ DNA/*HindIII* Marker containing 5 µL of dye. The gel was run at 50-60 V and when the bromophenol blue tracking dye migrated down two thirds the length of the gel it was turned off. The gel was immediately post-stained with Ethidium Bromide (12.5 µL EtBr dissolved in 100 mL 0.5 X TBE buffer) and placed on a bench top shaker for 45 minutes. An image of the gel was taken using a camera in the lab.

EcoRI Digestion of Cloned Plasmids

To cut the vector just to each side of the insert, aliquots of the miniprep samples were digested with EcoRI. Digestions were run in eppendorf tubes. Into each eppendorf tube, 39 µL Autoclaved, distilled water, 5 µL NEB Buffer (10X), 5 µL of plasmid containing approximately 1 µg of DNA (*yj15*, *yj13*a, *yj13*b, or *yj41*), and 1 µL EcoRI were added in that order to reach a total volume of 50 µL. The tubes were incubated overnight at 37°C.

Sample Digests Agarose Gel Electrophoresis

To determine whether the insert was the correct size, agarose gel electrophoresis was performed on the digested DNA using a 2% agarose gel, according to the procedure for the PCR samples.

Nanodrop Measurement of DNA concentrations

For sequencing by Eton Bioscience, each sample should contain 10 µL of 100 ng/µL plasmid DNA. To make sure the required amount of DNA was available for sequencing, the DNA concentration was measured using a Nanodrop 2000

instrument. To use the Nanodrop, the sampling arm was raised and 1µL of a DNA sample was pipetted onto the lower measurement pedestal. Lowering the sampling arm initiated a spectral measurement using the software on the PC. The sample column was automatically spread between the upper and lower pedestals and the measurement was made. When the measurement was complete, the sampling arm was raised and the sample was wiped from both the upper and lower pedestals using a dry Kimwipe.

Preparing samples for sequencing

If plasmid samples had been eluted from the Spin Prep columns in water, the concentrations could simply be adjusted to 100 ng/µL to be sent out for sequencing. However, for samples eluted in buffer EB, it was necessary to transfer the samples to water. This was accomplished by adding enough ethanol to fill the 1.5 mL sample tube, thereby precipitating the plasmid DNA. The sample was kept overnight at -20° C. The next day, the DNA was pelleted for 10 minutes in a microcentrifuge at 13,000 x g. The supernatant was removed with a pipet tip, and the pellet was allowed to air dry. Finally, the DNA was redissolved in an appropriate volume of sterile distilled water to make a 100 ng/µL solution.

Chapter 3. Results

The goal of this project was to identify and confirm genetic mutations that are within the *srf-6* sequence. To accomplish this task, the entire genomes of three *srf-6* mutants were sequenced (Igor Antoshechin, California Institute of Technology, unpublished). These were mutants, 4A.2, O2.2, and C.2 , which are commonly referred to as *yj41*, *yj15,* yj*13* respectively in this report. The whole genome sequencing was initially done before our project started; in that work, with a different variant selection method, an initial *srf-6* candidate, F41G3.2, was chosen (S. M. Politz, unpublished). The investigation on the first candidate, F41G3.2, was completed utilizing the same protocols (Materials and Methods) as were used for the *nsy-1* candidate gene described later in this section. In summary this data was not consistent with what was expected because although there were mutations present, they were not where Galaxy had found the mutations in the whole genome sequences. Consequently, further investigation of the F41G3.2 candidate was not pursued.

Srf-6 Mapping by Recombination

The *srf-6* genetic sequence has been mapped to the chromosome II region of the *C. elegans* genetic map via recombination mapping. While initially this was interpreted to be within 6,600,000-6,800,000 basepair range, it is possible that the *srf-6* sequence is located to the left of *dpy-10* due to one deficiency noted when the mapping was complete, as well as three factor crossing which indicated that *srf-6* was to close to *dpy-10* on the right or left (Grenache et al., 1996).

Early Work: F41G3.2

Based on the mapping of *srf-6,* our lab had the whole genome sequences of three *srf-6* mutants determined, for the purpose of identifying mutant DNA sequence changes corresponding to *srf-6.* The rationale was that the independently isolated *srf-6* mutants should all have sequence changes in the same gene. The fact that the *srf-6* mutations failed to complement each other, and all mapped to chromosome II, suggested that they all affected the same gene, which we should be able to find by analysis of chromosome II DNA sequences. The online software package Galaxy was used to align the three mutant sequences with the published wild-type *C. elegans* genome according to the workflow described in (Minevich et al., 2012). Variant sequences that differed from wild-type were identified, and a subtraction method was used to remove identical variant sequences shared in common by the mutants. The remainder of the variant sequences should include *srf-6* mutations. This list of variants served as a starting point for our project.

A gene was found in chromosome II that contains mutations found in *srf-6* mutant DNAs. This protein-coding gene, *F41G3.2*, is 268 amino acids long and extends from 6,759,291 bp - 6,760,315 bp. The position of the F41G3.2 mutations differs among the mutant strains (listed in Table 1). All three mutations are in coding sequences. In the *yj15* strain, a frameshift mutation where a T is inserted at the 6,759,927 bp position is found. In the *yj41* strain a missense mutation is found at 6,759,930 bp of CAA to GAA, which changes a glutamine codon (Q) to a glutamic acid codon (E). A missense mutation is also found in the *yj13* strain, where at position 6,759,927 bp CCC is changed to TCC, changing a proline codon (P) to a serine

codon(S). The mutations are all within 3 bp of each other. Because F41G3.2 is a protein coding gene, and due to it being within the region where *srf-6* is suspected to be, this makes it a good candidate.

Strain	Mutation Site	Mutation Type	Sequence Change
yj41	6,759,930	Missense	CAA=>GAA
yj13	6,759,927	Missense	CCC=>TCC
yj15	6,759,927	Frameshift	T insertion

Table 1: F41G3.2 Mutations obtained from the whole genome sequencing of *srf-6* mutants

Re-sequencing of F41G3.2 candidate mutations

To confirm the whole genome sequencing results, we cloned PCR products, using one primer pair, that should have included each of the three mutant sites. In **Figure 3** we see that all the different PCR products amplified with the same primer pair migrate just below the 564 bp mark, which is where the F41G3.2 PCR products should be located.

Figure 3: F41G3.2 PCR products with *yj41*, *yj13*, *yj15* DNA templates.
Lane 1: Lambda HindIII Lane 2: *yj13*, Lane 3: *yj15*, Lane 4: *yj13*

```
WT  6759736 CGTATCATCTCAGTGGAGCATATGGAAATGCAAATTATGGTTCAATGCCAAGAGCT
yj41 6759736 CGTATCATCTCAGTGGAGCATATGGAAATGCAAATTATGGTTCAATGCCAAGAGCT
yj13 6759736 CGTATCATCTCAGTGGAGCATATGGAAATGCAAATTATGGTTCAATGCCAAGAGCT
yj15 6759736 CGTATCATCTCAGTGGAGCATATGGAAATGCAAATTATGGTTCAATGCCAAGAGCT

WT  6759792 CAACGAAAGCCAGGTTTTTTTTTTAATTTTCAGAGAGATGTTTCCATTGGAACAAG
yj41 6759792 CAACGAAAGCCAGGTTTTTTTTTTA  TTTTCAGAGAGATGTTTCCATTGGAACAAG
yj13 6759792 CAACGAAAGCCAGGTTTTTTTTTTA  TTTTCAGAGAGATGTTTCCATTGGAACAAG
yj15 6759736 CAACGAAAGCCAGGTTTTTTTTTTA  TTTTCAGAGAGATGTTTCCATTGGAACAAG

WT  6759848 AATATAAAACTTATCAATTCAGGATCTTACCAACCTCAAATGCATGATCATCGTTC
yj41 6759848 AATATAAAACTTATCAATTCAGGATCTTACCAACCTCAAATGCATGATCATCGTTC
yj13 6759848 AATATAAAACTTATCAATTCAGGATCTTACCAACCTCAAATGCATGATCATCGTTC
yj15 6759848 AATATAAAACTTATCAATTCAGGATCTTACCAACCTCAAATGCATGATCATCGTTC

WT  6759904 ACAAGACGGAGGATACCGTCAATCCCCGAA  CCGA
yj41 6759904 ACAAGACGGAGGATACCGTCAATCCC  GAAACGGA
yj13 6759904 ACAAGACGGAGGATACCGTCAATCCC  GAAACGGA
yj15 6759904 ACAAGACGGAGGATACCGTCAATCCC  GAAACGGA
```

Figure 4: F41G3.2 Re-sequencing alignment of *yj41*, *yj15*, yj*13*.
Mutations are described in the text.

In **Figure 4**, F41G3.2 resequencing results, which start at position 6,759,736 bp, revealed that the PCR products did not contain the F41G3.2 mutations that had been found by whole genome sequencing. Instead, several mutations were found that were the same in all three mutants. The first was a deletion of an A at 6,759,818. A second mutation was a deletion of a C at 6,759,931. Finally, an A insertion was found at 6,759,935. There was only one usable sequence for *yj41*, but the mutation found in the whole genome sequence was not present. For *yj13*, two sequences were compared with the reference genome but the mutations found in the whole genome sequence were not found (only one sequence shown). In *yj15* two cloned insert sequences were aligned with the reference genome, but there was a T deletion in one of them that made the two sequences unaligned (deletion not shown in figure). Based on these results, further analysis of the F41G3.2 *srf-6* candidate was abandoned.

Later Work: nsy-1

Galaxy Project Sequencing

To facilitate further analysis of the whole genome sequence data, an additional step was added to the workflow. Using the assumption that all three *srf-6* mutations should be in the same gene, the Galaxy in-silico complementation test plug-in was used to identify mutations from all three mutants that were common to a single open reading frame in the reference genome.

Sample Name	Chromo #	Position	Ref.	Change	Change type	Homo/ Hetero	Gene ID	Gene name	Bio type	Transcript ID	Effect	Old AA/ new AA
Data 10 C.2 (*yj13*)	II	5024741	G	A	SNP	Homo	nsy-1	protein coding	F59A6.1	Exon IV: 5024491-5024885	D/N	**G**at/**A**at
Data 32 4A.2 (*yj41*)	II	5026465	G	A	SNP	Homo	nsy-1	protein coding	F59A6.1	Exon VII: 5026317-5027231	G/E	g**G**a/g**A**a
Data 20 O2.2 (*yj15*)	II	5026995	G	A	SNP	Homo	nsy-1	protein coding	F59A6.1	Exon VII: 5026317-5027231	D/N	**G**at/**A**at

Table 2: *nsy-1* Mutation Information Obtained from Galaxy Program via In-Silico Complementation. Under Sample name, the appropriate *srf-6* alleles are listed. The locations of each mutation are shown in the "Position" column. The reference sequence is shown in column Ref., and the mutant sequence change is shown in column Change. The Homo/Hetero column refers to whether the whole genomic sequence results contained evidence that a genome was homozygous or heterozygous for the identified sequence change. Exons shown in the Transcript ID column are from the UCSC genome browser. The Effect column shows the amino acid change that results from each mutation. The last column shows the reference and mutant codon sequences for each mutant.

From the resulting in-silico complementation tests in the Galaxy Platform, *nsy-1*appeared as a candidate. While there were other candidates in the chromosome II region where *srf-6* has been mapped to, *nsy-1* was the only protein coding gene. The other possible mutations were located in introns, they were not in the right positions in chromosome II, or they only contained two of the mutants. All three mutants contained non-synonymous substitutions in the *nsy-1* coding sequence. As described in **Table 2**, these mutations had many features that made *nsy-1* a feasible *srf-6* candidate sequence. Each *srf-6* mutant contained a homozygous mutation in *nsy-1*, which is preferable over heterologous mutations. Because the strains subjected to whole genome sequencing were homozygous for the *srf-6*

mutation, any candidate resulting from whole genome sequencing should also be homozygous. *nsy-1* was a protein coding gene that was located on the chromosome II to the left of *dpy-10*. As noted in the introduction, this was within the acceptable range for the *srf-6* sequence.

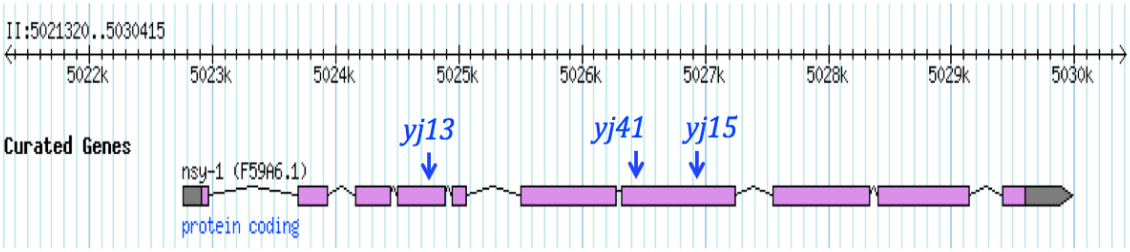

Figure 5: Intron-Exon map of *nsy-1* showing the locations of the putative mutation sites.
The ruler line above shows the corresponding positions in the physical map of chromosome II (compare with the nucleotide positions in Table 1).

Based on the data given by **Table 2**, the *yj13, yj15,* and *yj41* mutations have all been mapped in the *nsy-1* sequence. The *yj13* mutation mapped to the fourth exon, while *yj41* and *yj15* both mapped to the seventh exon.

PCR amplification of genomic DNA containing the putative *srf-6* mutations.

PCR primers were designed and applied to the appropriate genomic template DNAs (Materials and Methods). PCR products were tested for quantity and proper size by agarose gel electrophoresis.

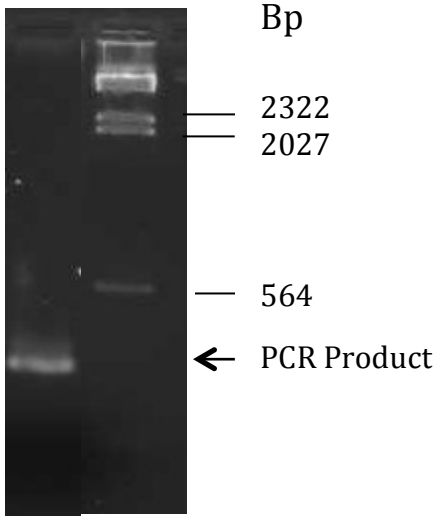

Figure 6: 2% agarose, 0.5X TBE Gel Electrophoresis of *yj15* PCR product.
Left lane: *yj15* Primers with *yj15* template, Right lane: λ DNA/*HindIII* Marker

In **Figure 6**, we see one definitive PCR product in lane two. The position of this band, below the 564 bp Lambda HindIII marker, is consistent with the expected size of 291 bp.

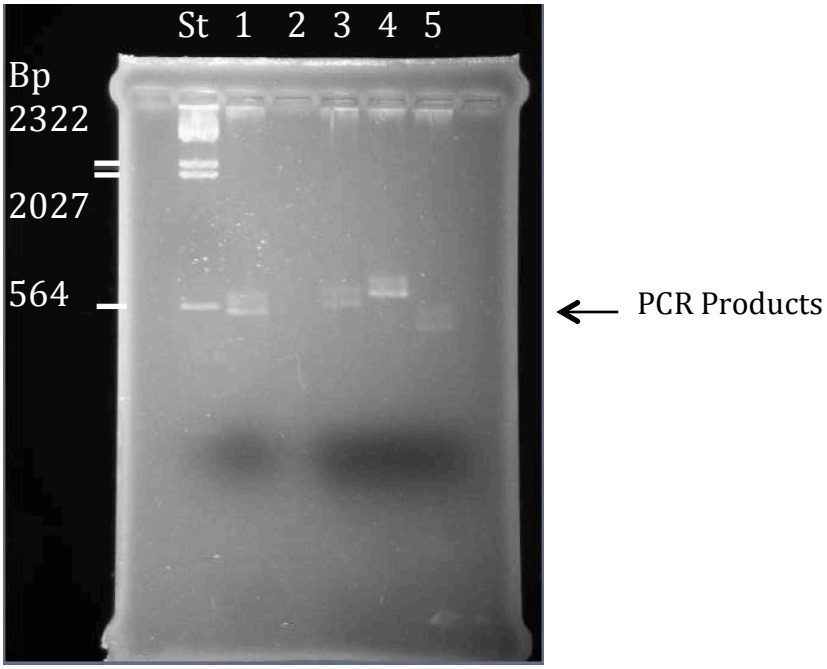

Figure 7: 2% Gel Electrophoresis for *yj41*a, *yj13*A, and *yj13*B.
Lane St: Lambda HindIII ladder, Lane 1:F41G3.2 with *yj41* Template, Lane 2: Blank, Lane 3: *yj13* A primer with *yj13* template, Lane 4: *yj13* B primer with *yj13* template, Lane 5: *yj41* primer with *yj41* template.

In **Figure 7**, bands of the appropriate size were obtained (near the 564 bp marker). Expected sizes were 545 bp for *yj13*A, 675 bp for *yj13*B, and 485 bp for *yj41*). The F41G3.2 primer was used as a positive control with a previously analyzed DNA template *yj41*.

Nanodrop Measurement of Plasmid DNA concentration

PCR products were cloned as described in Materials and Methods. A Nanodrop instrument was used to quantify DNA concentrations of cloned plasmid DNAs.

Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type
	7.8	ng/µl	0.156	0.062	2.52	2.26	DNA
02.2.1	10.7	ng/µl	0.213	0.095	2.24	1.37	DNA
02.2.2	1.3	ng/µl	0.025	-0.001	-43.42	-0.83	DNA
02.2.3	4.8	ng/µl	0.096	0.042	2.25	0.88	DNA
4A.2.1	10.4	ng/µl	0.208	0.097	2.15	1.68	DNA
4A.2.2	6.0	ng/µl	0.120	0.048	2.48	7.12	DNA
4A.2.3	5.6	ng/µl	0.113	0.042	2.68	6.46	DNA
C.2.1	9.9	ng/µl	0.199	0.099	2.00	1.19	DNA
C.2.2	1.9	ng/µl	0.038	0.006	6.27	-1.95	DNA
C.2.3	1.5	ng/µl	0.030	0.003	10.60	-1.19	DNA
C.2.B.1	0.3	ng/µl	0.006	-0.011	-0.56	-0.28	DNA
C.2.B.2	4.5	ng/µl	0.091	0.032	2.81	8.38	DNA
C.2.B.3	2.5	ng/µl	0.050	0.012	4.00	-2.69	DNA

Table 3: DNA concentrations of cloned plasmid DNAs.
Each row indicates a single cosmid clone. Three colonies from each cloning reaction were grown separately and DNA isolated.

In **Table 3**, many of the 260/280 ratios are above 2.00, indicating that the DNA could be contaminated. Furthermore, the absorbance at the A260 wavelength in all samples indicated that DNA concentrations were too low for sequencing. Eton Bioscience requires 10 µL of a 100 ng/µL solution for sequencing (http://www.etonbio.com/).

Chapter 4. Discussion

The objective of this project was to locate and confirm *srf-6* mutations s to

facilitate the study of *srf-6* function. To accomplish this task, after whole genome sequencing of the three mutant strains, variant sequences from the three *srf-6* mutant strains were analyzed using Galaxy to determine whether mutations in a specific gene are found in all three mutant DNA sequences.

Initially the first candidate, F41G3.2, was considered to be a prospect for *srf-6*. However, after reviewing the re-sequenced data from Eton Biosciences (**Figure 4**), we found that the expected mutations, according to Galaxy, were not present in their supposed positions, and conversely, mutations were found in the resequenced cloned DNA that were not found in the whole genome sequences of the *srf-6* mutants. This could be for several reasons. A possible reason for the lack of mutations could have been a misread during the whole genome sequencing or the plasmid insert sequencing. For the cloned sequences, sequence changes could have occurred during PCR amplification of DNA. Due to the failure of any of the mutations in F41G3.2 to be reproduced, it was decided that locating another *srf-6* candidate and re-sequencing it was the next step. It was decided that a different variant selection process would be needed.

In the resulting in-silico complementation test, *nsy-1* was one attractive candidate gene detected by Galaxy. While there were other genes in the region with mutations found by in-silico complementation, *nsy-1* had additional traits that added to its case as a *srf-6* candidate, shown in **Table 2.** *nsy-1* is a protein-coding gene, which coincides with the fact *srf-6's* suspected identity is also a protein-coding gene. *nsy-1* encodes a MAP kinase kinase kinase and is known to play a role in chemosensation (Sarvasti et al, 2011). The *srf-6* mutation has shown to result in

chemotaxis defects, so the overlap in roles could potentially point to *nsy-1* as being the same gene as *srf-6. nsy-1* is located to the left of *dpy-10,* which is in the realm of possibility for being *srf-6* due to deficiency mapping and three-factor crosses that indicated *srf-6* could also be to the left of *dpy-10* (Grenanche et al, 1996). Furthermore, *nsy-1* has been previously studied and stocks of *nsy-1* mutants were readily available for experimentation. In worms, pair of bilateral olfactory neurons, AWCL and AWCR, are found in the amphid neurons in the head. Only one of these neurons expresses *str-2*, an olfactory receptor, in wild type *C. elegans*. A properly functioning *nsy-1* gene product is required for this asymmetric expression. When *nsy-1* is disrupted, *str-2* is expressed in both neurons (Sagasti et al, 2001).

nsy-1 encodes ASK-1, a protein homologous to a human MAPKKK that has roles in several processes including regulation of apoptosis signals (Ichijo et al., 1997). In *C. elegans*, the role of *nsy-1* is downstream of the CaMKII homolog, *unc- 43*, in the pathway that controls cell fate in AWC. It is predicted that in *C. elegans* CaMKII UNC-43 activates the NSY-1 MAPKKK through a voltage-gated calcium channel. *nsy-1* and *unc-43* mutants are both implicated in the establishment of AWC asymmetry and they both express the same *str-2* expression phenotype (Sagasti et al., 2001). In *C. elegans*, *nsy-1* is expressed in the intestine, hypodermis, rectal gland cells, and neurons. *nsy-1*'s presence in multiple areas of the worm suggests that it could have control over other functions besides str-*2* asymmetry (Ichijo et al., 1997). *nsy-1* is 1498 residues long. Its catalytic region ranges from 669-925 amino acid residues within the protein. This translates to 768 total bp, starting at 5,026,455 bp and

ending at 5,027,222 bp. This region is included in most of the seventh exon of *nsy-1*. It was found that only the yj15 mutation at position 5,026,995 was encapsulated within the catalytic domain. All the other mutations lay outside of this area.

Overall, the Nanodrop results indicate that there is not enough DNA for us to send out the *nsy-1* primer products for sequencing. Eton Biosciences requires 100 ng/μL DNA in order for sequencing to be done. None of our samples contained enough DNA for sequencing alone. This issue could be due to a change in protocol. A vacuum manifold was used to purify and obtain DNA samples for *nsy-1*. This was not done for the F41G3.2 plasmid isolations, which were successful, and in future experiments we recommend returning to the centrifuge protocol to purify DNA. Another approach is to test the *nsy-1* mutants for *srf-6* phenotype and test the *srf-6* mutants for *nsy-1* phenotype. To test the *nsy-1* mutants for the *srf-6* phenotype, immunofluorescence testing could be performed using monoclonal antibody M37, as was done in Grenache et al 1996. If the *nsy-1* mutants expressed the L1 epitope (which is recognized by M37), at later larval stages it would be shown by fluorescence at stages L2-L4. A test to see whether the *srf-6* mutants have the *nsy-1* phenotype is to use a GFP-tagged *str-2* transgene to see if they express *str-2* in both AWCL and AWCR neurons, as described in Sagasti et al. (2001). To test whether the *srf-6* and *nsy-1* mutations affect the same genetic function, a complementation test could be performed. By crossing homozygotes from *srf-6* and *nsy-1* mutant strains, and testing their heterozygous offspring for the *srf-6* and *nsy-1* phenotypes, it can be determined whether the two mutations affect the same function or two different functions. If the resulting heterozygotes are wild type, then *nsy-1* and *srf-6* are

different genes. However, if the offspring continue to exhibit traits inherent to the respective mutations, then *nsy-1* and *srf-6* are in fact within the same genetic sequence.

The purpose of our project was to identify mutations that correspond with the *srf-6* gene to enable future study. This is important because the surface antigen switching phenotype of *srf-6* in *C. elegans* is known, however its location within the genome is still unknown. Identifying a gene within the genome that corresponds to *srf-6*, its location could be mapped allowing future study and manipulation of that gene to be possible.

C. elegans is suitable as a model for parasitic worms due to their high degree of similarity in cuticle structure, development, and surface composition. Once the *srf-6* DNA sequence is identified, a sequence search for similar genes in parasitic nematodes can be conducted. If parasitic nematodes contain such a gene, it could serve as a target for design of an anti-nematode control drug that inhibits *srf-6.* could lead to a drug could be found that inhibits nematode *nsy-1*/*srf-6.* In countries where parasitic infections are prevalent this is of high importance because if a drug were found that inhibits surface antigen switching in these parasitic worms it might improve the ability of host defenses to attack the parasite. Therefore, understanding the *srf-6* mutation has a high applicability in areas of human health.

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